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The purpose of this research project is to better understand the interaction of dietary supplements with cancer chemotherapeutic drugs. This information may be useful to decrease the toxicity and increase the effectiveness of chemotherapy. The scope of the research involves *in vivo* assessments in rats of nutritional supplement-chemotherapeutic drug interactions and *in vitro* studies of the mechanisms of nutraceutical-chemotherapeutic drug interactions. Vitamin E and hyperforin levels in rat plasma correlated with dietary intake. There was no significant effect of vitamin E supplementation on the hematologic toxicity or survival in rats treated with a range of doxorubicin or docetaxel doses. There were no important perturbations of the pharmacokinetics of doxorubicin related to dietary intake of vitamin E or St. John's wort. Rats ingesting St. John's wort had similar survival at each dose level of doxorubicin to rats ingesting the control diet. There were no important modulatory effects of vitamin E on mitochondrial DNA damage after doxorubicin. Our studies indicate that vitamin E and St. John's wort neither increase nor protect against the toxicity of doxorubicin or docetaxel in rats.

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Table of Contents

INTRODUCTION

The general <u>subject</u> of this research project is the effects of dietary supplements on the pharmacokinetics and pharmacodynamics of chemotherapeutic drugs used to treat women with breast cancer. More specifically, the research focuses on the effects of St. John's wort as an example of a nutraceutical and Vitamin E as an example of a nutritional supplement on doxorubicin, docetaxel, and cyclophosphamide. The hypothesis to be tested is that nutritional supplements have important effects on the pharmacokinetics and pharmacodynamics of cancer chemotherapeutic agents. The <u>purpose</u> of the research is to better understand the interaction of dietary supplements with cancer chemotherapeutic drugs and then utilize this knowledge to alert patients and their physicians to these interactions. This information also may be useful to decrease the toxicity and increase the effectiveness of chemotherapeutic drugs. The <u>scope</u> of the research involves *in vivo* assessments in rats of nutritional supplement-chemotherapeutic drug interactions and *in vitro* studies of the mechanisms of nutraceutical-chemotherapeutic drug interactions.

BODY

Task 1. Evaluate the effects of supplementation with St. John's wort and vitamin E on the pharmacokinetics of cyclophosphamide, docetaxel and doxorubicin.

a. Establish and refine, as necessary, methodology for analysis of plasma concentrations of 4-hydroxycyclophosphamide, docetaxel, doxorubicin, vitamin E and hypericin.
b. Maintain 3 groups of rats on diet alone, or diet plus St. John's wort or vitamin E.
c. Inject rats with chemotherapeutic agents and collect plasma samples.
d. Measure drug levels in plasma.
e. Analyze pharmacokinetic data.
f. Repeat phamacokinetic studies with different doses.

a. Establish and refine, as necessary, methodology for analysis of plasma concentrations of 4-hydroxycyclophosphamide, docetaxel, doxorubicin, vitamin E and hypericin.

i. As reported last year, we refined the technique to measure α-Tocopherol (vitamin E) concentrations in plasma using a reverse-phase high-performance liquid chromatography method with UV detection as previously reported by Julianto *et al.* (1). During the past year we used this method to measure vitamin E levels in rats maintained on diets with differing vitamin E contents.

ii. Our original plan was to focus on analysis of hypericin as an indicator molecule for our pharmacological studies of St. John's wort. However, because some recent studies have identified hyperforin as a more important contributor to the pharmacological actions of this herbal product (2), we have employed an HPLC analytical method that accurately detects and determines hyperforin. Hyperforin concentrations in plasma were measured by reverse-phase high-performance liquid chromatography with UV detection. Briefly, hyperforin was extracted from plasma by solid-phase extraction on 3mL Strata C18E columns (Phenomenex, Torrance, CA). The columns were first conditioned with 3mL methanol followed by 3mL water. The samples were loaded onto individual Strata columns and washed with 2mL of water and hyperforin was eluted from the columns with 1mL of methanol. The eluents were evaporated

to dryness under nitrogen and the residues were reconstituted in 75% acetonitrile in water containing 3mL phosphoric acid per liter. The mobile phase consisted of acetonitrile-0.01M ammonium phosphate (pH 2.5) (85:15 [vol/vol]) with a flow rate of 1.5 mL/min through a Symmetry C18 5μ column with a matching guard column (Waters, Milford, MA). The detector was operated at a wavelength of 272nm and the samples were quantified using peak area. A representative standard curve is illustrated in Figure 1.

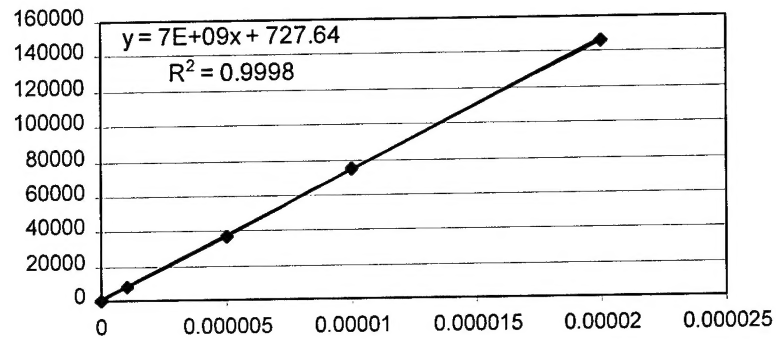

Figure 1. Hyperforin standard curve.

iii. As reported last year, doxorubicin concentrations in plasma were measured with a reverse-phase high-performance liquid chromatography method using fluorescence detection as previously reported by Warren *et al.* (3) and as modified by us.

iv. The method for assaying plasma docetaxel concentrations is currently being refined using a modification of the method described by Willey and coworkers (4). Briefly, plasma samples were buffered with 0.2M ammonium acetate (pH 5.0) and mixed. Docetaxel was extracted from plasma by solid-phase extraction on 1mL cyano Bond Elut (Varian, Walnut Creek, CA) columns. The columns were first conditioned with 2mL methanol followed by 2mL 0.01M ammonium acetate (pH 5.0). Samples were loaded onto individual Bond Elut columns and washed with 2mL of 0.01M ammonium acetate, 2 mL of 20% methanol in 0.01M ammonium acetate (pH 5.0), and 1 mL hexane. Docetaxel was eluted from the columns using two 1mL volumes of 0.1% triethylamine in acetonitrile. The eluents were evaporated to dryness under nitrogen and the residues were reconstituted in acetonitrile-methanol-0.01M ammonium acetate in water (pH 5.0) (4:1:5 [vol/vol]). The mobile phase consisted of 0.02M ammonium acetate (pH 5.0)-methanol-acetonitrile (10:2:8 vol/vol]) with a flow rate of 1.0mL/min through an Alltima C8 5μ column with a matching guard column (Alltech, Deerfield, IL). The detector was operated at a wavelength of 227nm and the samples were quantified using peak area.

b. **Maintain 3 groups of rats on diet alone, or diet plus St. John's wort or vitamin E.**

i. As reported last year, weanling female Fisher 344 rats, 4 or 5 rats per group, were fed either a cereal-based, standard rat diet that supports growth and maintenance (Harlan-Teklad Global,

Product # TD 00217) that contains 16% protein, 3.5% fat and 102 I.U./kg Vitamin E (α-tocopherol), or the same diet supplemented with either a high level of vitamin E, 750 I.U./kg (Harlan-Teklad Product # T.D. 01375) or a low level of vitamin E, 50 I.U./kg (Harlan-Teklad Product # T.D. 01374). During the past year, the plasma levels of vitamin E were measured and confirmed that dietary vitamin E content influenced plasma vitamin E levels. Thus, rats on the diet containing 102 I.U./kg vitamin E had plasma levels of 15.4 uM, those on the diet containing 152 I.U./kg vitamin E had plasma levels of 20.0 uM, and those on the diet containing 750 I.U./kg vitamin E had plasma levels of 26.2 uM. These values are similar to levels reported in the literature for rodents maintained on diets enriched for vitamin E (5).

ii. Last year we reported that initial exploratory studies were performed with a standardized preparation of St. John's wort (HBC St. Johns wort) that was used in clinical trials and pharmacokinetic studies supported by the National Institutes of Health. A suspension was administered daily to rats by gavage. Unfortunately, all of the animals lost weight, and most died before the completion of a planned 14 day course. A review of the ingredients of this St. John's wort preparation indicated that the excipients included silicon dioxide. We believe that this ingredient caused intestinal complications. However, several other St. John's wort preparations also caused toxicity when given by gavage. Further exploratory studies suggested that the stress of gavage in young rats contributed importantly to the observed mortality. During the past year we incorporated the St. John's wort into the diet rather than administer it by gavage. A custom diet was formulated that consists of Teklad Global 16% Protein Rodent Diet with 4 g of St. John's wort /kg of feed (4 mg/g). The St. John's wort preparation is Optical Nutrients product 14772 which consists of flowering tops and leaves and is standardized to 0.3% hypericin. Other ingredients include maltodextrin, rice powder, gelatin and magnesium sterate. A 150g rat ingests about 15 g of chow per day or 400mg/kg of body weight. This quantity of dietary St. John's wort has been calculated to approximate pharmacologically relevant doses of St. John's wort in humans (6). On this diet the rats grow at the same rate and ingest the same quantity of food as rats maintained on a control diet. The hematocrit and white blood cell counts measured after 2 weeks on the diet are not significantly different from rats maintained on the standard diet. After 2 weeks on the diet, the rats were found to have hyperforin levels of $2.50 \pm 0.69 \times ^{-6}$ M. This drug concentration is comparable to levels we measured in rats that were given St. John's wort by gavage ($1.38 \pm 0.32 \times ^{-6}$ M) and similar to levels of hyperforin reported in the literature by other laboratories (6). A lower dietary intake of 1 g of St. John's wort/kg of feed (1 mg/g) resulted in a hyperforin level of $2.04 \pm 0.69 \times ^{-6}$ M.

c. **Inject rats with chemotherapeutic agents and collect plasma samples.** We chose to begin with studies of the effects of St. John's wort and vitamin E on doxorubicin. These studies are essentially completed, and we have progressed to studies of docetaxel. All samples were obtained from the saphenous vein of the animals. Both lower hind legs on each animal were shaved. A thin layer of silicone grease was applied, and the leg was held with sufficient pressure to cause the vein to become clearly visible. Using a Microlance blood lancet a small puncture wound was made in the saphenous vein. The blood was collected in a StatSpin heparinized collection tube, mixed, and centrifuged at 12,000 rpm for 10 minutes. The plasma was removed, placed in microcentrifuge vials and stored at -80°C until testing. Blood samples were obtained prior to injection of the drug and at 10, 20 and 30 min, and 1, 3, 5, 7, 24 and 48 hrs. The results for doxorubicin are shown in Table 1.

d. Measure drug levels in plasma.

i. Table 1. Pharmacokinetics parameters of doxorubicin in control, vitamin E or St. John's wort-treated rats analyzed by Win-Nonlin.

Diet	AUC*	Cl (ml/min)	$T_{1/2}$ (min)	Vss (liters)	N¶
Control	6.1 ± 2.5#	32.45 ± 19.38	96.45 ± 28.04	4.5 ± 1.6	4
High E	7.4 ± 1.1	22.64 ± 4.45	132.32 ± 30.14	4.6 ± 1.0	5
Low E	8.5 ± 4.1	22.18 ± 12.14	86.17 ± 44.69	3.5 ± 0.7	4
St. John's wort	9.8 ± 3.9	20.92 ± 7.66	108.10 ± 31.48	3.7 ± 2.7	6

*AUC x 10 = micromoles/liter x minutes
¶ N = number of animals
# mean ± SD

ii. Pharmacokinetics of docetaxel in control, vitamin E or St. John's wort-treated rats. Animals were injected with 7mg/kg of docetaxel. Blood samples from rats on the control diet, the vitamin E supplemented diets, and the St. John's wort supplemented diet have been collected and frozen. They are awaiting HPLC analysis.

e. Analyze pharmacokinetic data. Data were analyzed by the WinNonlin software.

i. Vitamin E and doxorubicin. Area under the time-concentration curve (AUC), clearance (Cl), half-life (t½), or volume of distribution (Vd) as calculated by noncompartmental modeling did not reveal any significant differences.

ii. St. John's wort and doxorubicin. Statistical analysis is being performed.

f. Repeat phamacokinetic studies with different doses. In our initial experiments rats were injected intravenously with doxorubicin at a dose of 5 mg/kg, but we observed plasma concentrations at later time points that were at or below the limit of quantitation and thus increased the dose modestly to 7.5 mg./kg.

Task 2. Measure the effects of supplementation with St. John's wort and vitamin E on the toxicity of cyclophosphamide, docetaxel and doxorubicin.

a. Maintain 3 groups of rats on diet alone, or diet plus St John's wort or vitamin E.
b. Administer chemotherapeutic drugs in LD50 doses.
c. Observe for toxicity and collect blood samples.
d. Analyze blood samples for evidence of hematologic, renal, hepatic and cardiac toxicity.
e. Analyze toxicity data.
f. Repeat toxicological studies with different doses.

i. The work is proceeding as outlined in Task 2. Last year, we reported that weanling female Fisher 344 rats were maintained on the same diets described in Task 1; that is, cereal-based or supplemented with a low dose (50 I.U./kg) or a high dose of vitamin E (750 I.U./kg). The rats grew at the same rate on all 3 diets. After 8 weeks the rats were

injected with increasing doses of doxorubicin (5.0, 7.5, 9.8, 12.8 and 17.9 mg/kg). The LD50 of doxorubicin was approximately the same for all 3 diets (12.7-13.2 mg/kg), and there was no dose-response relationship for vitamin E. Measurements of hematocrit and white blood cell counts at Days 4, 9 and 14 following injection of doxorubicin showed no important differences among the dietary groups.

ii. Rats maintained on the same vitamin E supplemented diets were injected with increasing doses of docetaxel: 8.5, 12, 18 and 20 mg/kg. Figure 2 shows that the LD50 of docetaxel was approximately the same (18 mg/kg) for all 3 diets. There was no significant difference in weight loss or hematologic toxicity among the dietary groups.

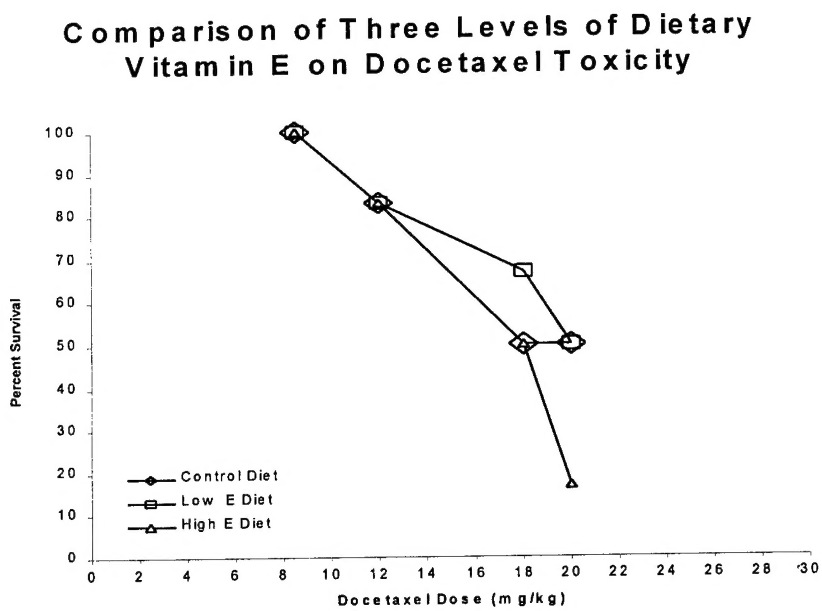

Figure 2. Effect of dietary vitamin E levels on docetaxel toxicity in rats.

iii. Rats were maintained on either a control diet or the same diet supplemented with 0.4% St. John's wort, as described in Task 1. After 2 weeks, the rats were injected with 12, 15, 18 or 22 mg/kg of doxorubicin. The results are illustrated in Figure 3. There were no striking differences in LD50, weight loss or hematologic toxicity between the two dietary groups. Statistical analysis of the data including survival (Figures 4-7) is proceeding.

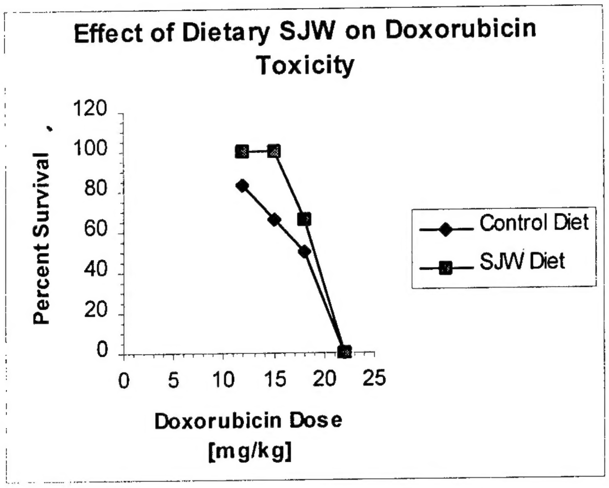

Figure 3. Effect of dietary St. John's wort on doxorubicin toxicity in rats.

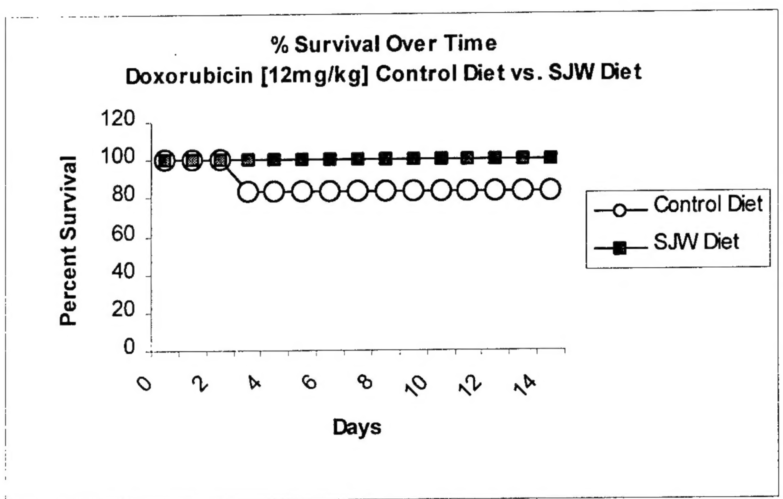

Figure 4. Effect of diet on survival after doxorubicin, 12 mg/kg, in rats.

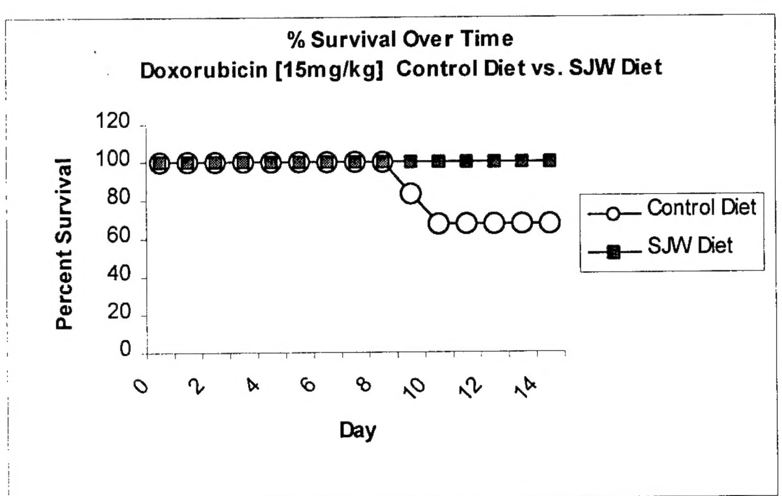

Figure 5. Effect of diet on survival after doxorubicin, 15 mg/kg, in rats.

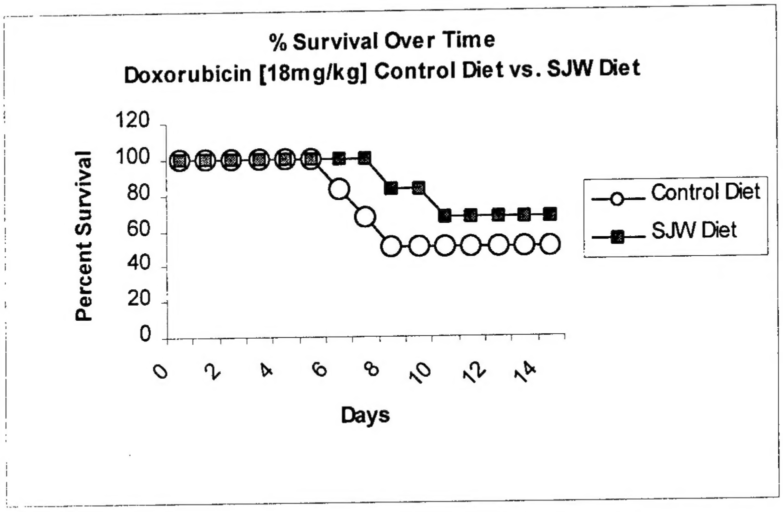

Figure 6. Effect of diet on survival after doxorubicin, 18 mg/kg, in rats.

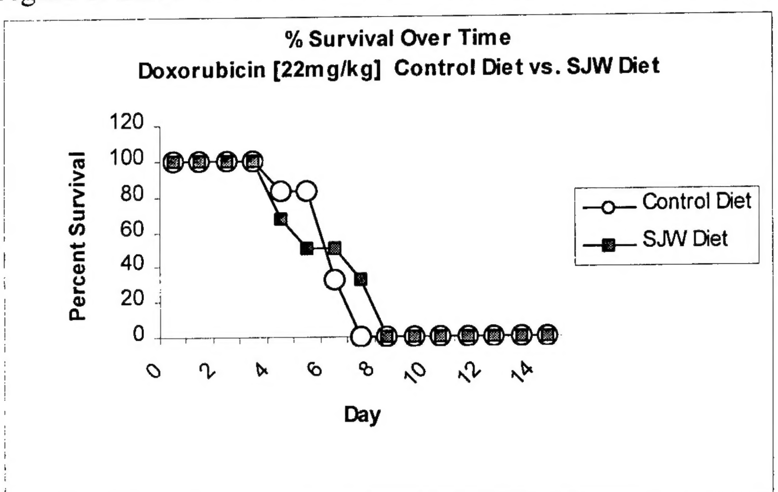

Figure 7. Effect of diet on survival after doxorubicin, 22 mg/kg, in rats.

Task 3. Study the mechanisms of nutraceutical-chemotherapeutic drug interactions.

a. Measure hepatic p450 activity where appropriate.
b. Measure hepatic P-glycoprotein expression where appropriate.
c. Measure glutathione S-transferase activity in liver samples where appropriate.
d. Collate and analyze data.

An important component of the toxicity profile of doxorubicin is cardiac toxicity. Prior studies have suggested that vitamin E supplementation may ameliorate this toxicity (7). Therefore we have initiated studies of the modulatory effect of dietary vitamin E on mitochondrial genetic damage caused by doxorubicin in rat heart and liver. A quantitative PCR (TaqMan) assay was developed to detect both mitochondrial DNA copy number and deletion frequency in the rat. This methodology allows not only the determination of changes in the amount of mitochondrial DNA deletion relative to total mitochondrial DNA but also to determine changes

in total mitochondrial DNA relative to genomic DNA (8). Table 2 shows the results obtained with this assay at a doxorubicin dose level of 12.8 mg/kg. The technique is described in detail in the appended manuscript (8).

Table 2. Mitochondrial DNA deletion and copy number in rats maintained on stardard rat chow or supplemented with low or high concentrations of vitamin E.

	Heart (mean ± SD)		**Liver** (mean ± SD)	
	ΔC_T Deletion	ΔC_T Copy Number	ΔC_T Deletion	ΔC_T Copy Number
Standard Diet	7.95 ± 0.62	12.33 ± 0.45	7.24 ± 0.29	10.01 ± 0.22
Doxorubicin				
Standard Diet	9.84 ± 2.80	12.34 ± 1.18	7.70 ± 0.97	10.73 ± 0.50
Low Vitamin E	12.45 ± 3.47	12.45 ± 1.82	8.12 ± 0.04	10.47 ± 0.14
High Vitamin E	9.96 ± 0.72	12.25 ± 0.40	8.08 ± 0.23	9.99 ± 0.32

In this experimental system, a smaller ΔC_T deletion indicates *more* deletions, while a smaller ΔC_T copy number indicates *less* total mitochondrial DNA. The data in Table 2 suggests that doxorubicin does not increase the number of deletions or copy number in cardiac or hepatic mitochondrial DNA, and that vitamin E supplementation does not modulate mitochondrial DNA damage.

KEY RESEARCH ACCOMPLISHMENTS DURING THE PAST YEAR

- High performance liquid chromatography (HPLC) methodology has been adapted/refined for the detection and determination of hyperforin and docetaxel.

- Vitamin E levels were measured in rat plasma and found to correlate with dietary levels of the vitamin.

- A diet supplemented with St. John's wort was formulated. The diet was palatable to the rats and supported growth at the same rate as the standard rat chow. Hematologic values after 2 weeks on the diet were not significantly different from levels in rats maintained on the standard diet. Measurement of hyperforin levels indicated that the diet resulted in plasma concentrations reported by other investigators to be pharmacologically relevant in rats and humans.

- Statistical analyses of the effects of dietary vitamin E on the pharmacokinetics of doxorubicin showed that area under the time-concentration curve (AUC), clearance (Cl), half-life (t½), or volume of distribution (Vd) as calculated by noncompartmental modeling did not reveal any significant differences.

- Analyses of the effects of dietary supplementation with St. John's wort on the pharmacokinetics of doxorubicin similarly show no dramatic differences. Statistical analysis is being performed.

- Samples have been collected and frozen for pharmacokinetic analyses of the effects of vitamin E and St. John's wort on docetaxel. These analyses are proceeding.

- Under the conditions used by us, we found that there was no significant effect of vitamin E supplementation on hematologic toxicity or survival in rats treated with a range of docetaxel doses.

- The LD50s for doxorubicin were similar in rats supplemented with St. John's wort and rats fed the standard chow. There was no striking difference in weight loss or hematologic toxicity in the two dietary groups. The statistical significance of these differences is being analyzed.

- Measurement of mitochondrial DNA deletions and copy number in rat heart and liver indicated that deletions tended to decrease after doxorubicin treatment while copy number was unchanged. There was no important modulatory effect of dietary vitamin E levels.

REPORTABLE OUTCOMES

Funding applied for based on work supported by this award:

- American Society of Clinical Oncology (ASCO) Young Investigator Award to Zafer Yildirim, MD, PhD, for a post-doctoral fellowship to work on this project was awarded.

Publications supported by this award:

- Yildirim, Z., Branda, R.F., Powden, C., Brooks, E., McCormack, J.J. Toxicity and pharmacokinetics of doxorubicin in rats treated with vitamin E. ASCO Proceedings, in press.

- Nicklas, J.A., Brooks, E.M., Hunter, T.C., Single, R., Branda, R.F. Development of a quantitative PCR (TaqMan) assay for mitochondrial DNA copy number and the common mitochondrial DNA deletion in the rat. Submitted.

CONCLUSIONS

The experiments described in this Annual Report analyze the relationship between dietary supplements and chemotherapeutic drugs used to treat patients with breast cancer. Herbal medicines and dietary supplements are used frequently by patients with cancer, but there is a paucity of information on their interactions with prescribed drugs. Since a majority of women with breast cancer are taking dietary supplements, there is a pressing need to better understand the effects of these supplements on cancer chemotherapy. Our studies suggest that even relatively high doses of vitamin E do not adversely affect the toxicity of doxorubicin or docetaxel. On the other hand, preliminary results suggest that St. Johns wort may decrease the toxicity of doxorubicin. Further studies will help to determine whether important interactions occur between these nutrients and cancer chemotherapeutic agents.

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DEVELOPMENT OF A QUANTITATIVE PCR (TaqMan) ASSAY FOR MITOCHONDRIAL DNA COPY NUMBER AND THE COMMON MITOCHONDRIAL DNA DELETION IN THE RAT

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Short Running Title: Quantitative PCR of mtDNA

ABSTRACT

Changes in mitochondrial DNA copy number and increases in mitochondrial DNA mutations, especially deletions, have been associated with exposure to mutagens and with aging. Common deletions that are the result of recombination between direct repeats in human and rat (4977bp and 4834bp, respectively) are known to increase in tissues of aged individuals. Previous studies have used long distance PCR and Southern blot or quantitative PCR to determine the frequency of deleted mitochondrial DNA. A quantitative PCR (TaqMan) assay was developed to detect both mitochondrial DNA copy number and deletion frequency in the rat. This methodology allows not only the determination of changes in the amount of mitochondrial DNA deletion relative to total mitochondrial DNA but also to determine changes in total mitochondrial DNA relative to genomic DNA. As a validation of the assay in rat liver, the frequency of the common 4834bp deletion is shown to increase with age, while mitochondrial DNA copy number decreases from birth to 3 months and then slowly increases.

Key words: rat, mitochondrial DNA, quantitative PCR, mitochondrial deletion, aging

INTRODUCTION

Somatic mutations in mitochondrial DNA (mtDNA) have been hypothesized to be a (or the) cause of aging and age-related disease (Harman, 1972; Linnane et al., 1989; Gadaleta et al., 1998). According to the 'free radicals theory of aging,' mitochondrial damage leads to free radical production that in turn causes further damage. Numerous studies have found increases in mitochondrial mutations in a variety of tissues from aged individuals. While point mutations of mtDNA have been shown to vary in both normal and malignant tissues and with age (Zhang et al., 1998; Fliss et al., 2000; Yowe and Ames, 1998; Kirches et al., 2001; Jones et al., 2001; Munscher et al., 1993; Penta et al., 2001), most studies have focused on the induction of large deletions in mtDNA. In humans, a 13bp direct repeat (at 8470-8482 and 13,447-13,459) in the mtDNA leads to the frequent occurrence of a 4977bp deletion by recombination between the repeats (Holt et al., 1989; Schon et al., 1989; Tang et al., 2000). Heteroplasmic inheritance of this deletion causes Pearson syndrome or Kearns-Sayre syndrome (Holt et al., 1989, 1989; Schon et al., 1989; Rotig et al., 1991), but this deletion is also a frequent occurrence during aging.

A similar common mtDNA deletion occurs in the rat due to a 16bp repeat at 8103-8118 and 12937-12952; recombination between these repeats leads to a 4834bp deletion (Gadaleta et al., 1992; Edris et al., 1994). This deletion also has been shown to increase in aged rats (Yowe and Ames, 1998; Gadaleta et al., 1992; Nagley et al., 2001; Seidman et al., 1997; Kang et al., 1998; Filburn et al, 1996). It can be increased by some drugs (Nagley et al., 2001; Suliman et al., 2002) or stress (Sakai et al., 1999) and decreased by dietary restriction in at least in some tissues in rats (Kang et al., 1998). Other mtDNA deletions also are seen in aged rats (Van Tuyle et al., 1996).

Quantitative PCR (qPCR) generally is used to detect changes in gene expression (RT-qPCR) but also can be employed to detect amplifications or deletions in genomic DNA. Quantitation studies have been performed either with Northern blots, Western blots, Southern blots or quantitative PCR blots, all of which are tedious, technically demanding and involve the use of radioisotopes. Furthermore, the PCR methods use an endpoint determination that is not truly quantitative because of plateau effects.

Several methods have been developed using real-time PCR (either TaqMan or molecular beacons) to monitor either mtDNA copy number or the amount of the common deletion in human cells (Rodriguez-Santiago et al., 2001; Reynier et al., 2001; Gahan et al., 2001; Szuhai et al., 2001; Heid et al., 1996; Lim et al., 2001; Steuerwald et al., 2000). Rodriguez-Santiago et al. (2001) determined mtDNA copy number in the brains of Alzheimer patients using quantitative PCR of the mtND2 gene versus the nuclear 18S gene. Wong and Bai (2002) determined copy number in patients with mitochondrial disease. Reynier et al. (2001) determined absolute mtDNA copy number in oocytes. Gahan et al. (2001) used molecular beacons of the mitochondrial cytochrome b and nuclear CCR5 genes to determine mtDNA copy number in human monocytes and in the fat of AIDS patients. Szuhai et al. (2001) also used molecular beacons for the mitochondrial tRNAlys gene and the nuclear globin gene to determine heteroplasmy in MERRF patients using a $\Delta\Delta C_T$ method. Lastly, Heid et al. (1996) and Lim et al. (2001) used the mtDNA and nuclear beta actin gene in a TaqMan relative curve method to quantitate mtDNA copy number, while Steuerwald et al. (2000) quantitated mtDNA copy number in oocytes using a standard curve method and SYBR green. Two groups have reported TaqMan methods to quantitate the human common deletion. Koch et al. (2001) used a non-deleted region probe and a probe specific for the deletion to quantitate the deletion in human

keratinocytes, while Meissner et al. (2000) used similar probes to detect the deletion in human blood.

We wished to develop a fast and inexpensive method to measure changes in mtDNA copy number and the frequency of the common mtDNA deletion. Here we report a rapid TaqMan method that quantitates rat mtDNA as compared to a nuclear gene (β-actin) as well as quantitates the amount of the common deletion relative to the number of copies of the D-loop region.

MATERIALS AND METHODS

Animals and DNA Extraction

The control female Fisher 344 rat (#6776) was obtained from Charles Rivers Canada (St.-Constant, Quebec) and fed *ad libitum* a standard cereal-based rat chow (LM-485 Harlan Teklad, Madison, WI). For the aging experiments, Sprague-Dawley rats were utilized. The animals younger than 3 months were a gift from Dr. Deborah Damon who obtained the mothers from Harlan Teklad (Madison, WI). The older animals also were obtained from Harlan Teklad at the indicated age, housed one week and then euthanized. They also were fed the standard chow as above. The Sprague-Dawley rats that were old enough for gender to be determined were all male. The rats were sacrificed and the liver tissue removed and stored at -80°C until processing. Total hepatic DNA was isolated using the Qiagen DNeasy Tissue kit (Valencia, CA).

Oligonucleotide Primers and TaqMan Probe Design

Primers and probes for the rat D-loop and the rat mitochondrial deletion from the rat mitochondrial genome (GenBank accession X14848) and rat β-actin primers (GenBank V01217)

were designed using Primer Express software (PE, Foster City, CA). Primers and probes were synthesized and HPLC purified by the PE Oligo Factory (Foster City, CA). Sequence for the primers and probes can be found in Table I and Figure 1.

Real-time PCR

Mitochondrial deletion expression was quantified with a 5' VIC reporter and a 3' TAMRA quencher dye and D-loop expression with a 5' 6-FAM reporter and 3' TAMRA labeled quencher dye. PCR amplification was carried out in a 50~L reaction consisting consisting of 1X TaqMan Universal Master Mix (Perkin-Elmer Applied Biosystems, Foster City, CA) and varied concentrations of probe and varied concentrations of forward and reverse primers to optimjze reaction conditions (see Table II). Optimal reaction conditions were those concentrations that gave the maximum ΔR_n and minimum C_T. The final optimal reaction concentrations for the common mitochondrial deletion/D-loop assays (CD/DL) (50ul reaction) consisted of 1X TaqMan Universal mix, 200nM each mitochondrial deletion forward and reverse primers, 100nM each D-loop forward and reverse primers, and 100nM each mitochondrial deletion and D-loop probe and ~50ng of sample DNA. For the β-actin/D-loop (BA/DL) assays, final conditions (50ul reaction) consisted of 1X TaqMan Universal mix, 200nM each β-actin forward and reverse primers, 50nM each D-loop forward and reverse primers, and 100nM each β-actin and D-loop probe and ~50ng of sample DNA. The cycling conditions include an initial phase of 2 min at 50°C, followed by 10 min at 95°C, 40 cycles of 15 sec at 95°C, and 1 min at 60°C. Each sample was assayed in duplicate and fluorescence spectra were continuously monitored by the 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA) with sequence detection software version 1.6.3.

Analysis of Data

Data analysis was based on measurement of the cycle threshold (C_T) which is the PCR cycle number when the fluorescence measurement reaches a set value. Two types of experiments were performed. The first measured mitochondrial copy number versus nuclear copy number by amplification of the mitochondrial D-Loop versus the nuclear beta-actin gene (BA/DL) while the second measured the amount of mitochondrial deletion versus the mitochondrial D-Loop (CD/DL). The difference in C_T values was used as the measure of relative abundance (i.e., in BA/DL experiments, C_T (BA) – C_T(DL) was used as the abundance of the mitochondrial genome and in the MD/BL experiments, C_T(CD)-C_T(DL) was used as the abundance of the mitochondrial deletion). Descriptive statistics and plots were used to explore possible trends in ΔC_T values based on the day that the experiment was performed. Linear regression was used to characterize the strength of any trends to the extent that they were linear.

Plots of age (X) versus ΔC_T (Y) indicated a non-linear relationship between the two variables. A negative exponential model, $y = \beta_0 + \beta_1 2^{(-x/\theta)}$, of the following form was chosen to characterize the change in ΔC_T with age. This model was chosen because it describes the non-linear relationship (decay) for the deletion data well and because of the interpretation of the parameters in the model. For this model θ represents the amount of time to lose half of the remaining amount and β_0 is the ultimate ΔC_T value, or asymptote.

RESULTS

The objective was to develop an assay that could detect both changes in the amount of mtDNA in a cell (as compared to a known nuclear gene which has 2 copies/cell) and in the amount of the common mtDNA deletion as compared to the number of mitochondria. We chose to use a sequence in the rat β-actin gene as our nuclear gene control, a sequence in the mitochondrial D-loop as the measure of the number of mitochondria, and a sequence that represented the new fusion sequence present only in deleted mtDNA as a measure for the common deletion.

TaqMan primers and probes were designed to the rat mitochondrial D-loop region, across the common deletion, and to the rat β-actin gene (Figure 1). PCR reactions were performed with these primers using liver DNA isolated from a single control rat. The specificities of all three sets of primers were tested by demonstrating the correct size product on an agarose gel and by DNA sequencing which revealed the correct sequence (data not shown).

For quantitative PCR, it is important that the efficiencies of the PCR reactions be close to 100%; (i.e. the slope of the graph of C_T versus log [DNA] is –3.33). Figure 2 plots the results for the actin, D-loop and common deletion PCR reactions. All the slopes are close to the optimal -3.33, with values of -3.346, -3.241 and -3.314 for the nuclear beta actin, mitochondrial D-loop and mitochondrial common deletion reactions, respectively.

Several experiments were performed changing primer and probe concentrations to optimize the assay and to allow utilization of as little primer/probe per assay as possible for cost considerations. Table II shows the results of these experiments. Use of one primer at 50nM while the other was 200nM did decrease the C_T; however, use of 100nM versus 200nM probes did not have much effect on the results.

TaqMan experiments can be quantitated absolutely using a standard curve made with solutions with a known number of copies, quantitated relative to a known sample, or quantitated comparatively in the same reaction to another gene (an endogenous control). In the latter, called the $\Delta\Delta C_T$ method, a multiplex PCR of the experimental gene and a control gene is performed. For any one reaction, the C_T of the experimental gene is subtracted from the control gene to control for the amount of input DNA and reaction conditions. To compare different experimental conditions, the ΔC_Ts of the two reactions are subtracted ($\Delta\Delta C_T$) and the DNA concentration difference between the two experiments is given by $2^{-\Delta\Delta C_T}$.

We chose to use the $\Delta\Delta C_T$ method because it affords savings in reagents and time. However, in order to utilize this method, it is necessary to demonstrate that the ratio of the two C_T's stays constant over a wide concentration range. In practical terms, this means that the absolute value of the slope of the ΔC_T versus log input amount of DNA is less than 0.1. Figure 3 shows the plots for the β-actin/D-loop (BA/DL) and common deletion/D-loop (CD/DL) results. For confirmation, experiments were performed with each PCR separately and in multiplex. These reactions gave similar results at a given concentration whether the PCR was a singleplex or multiplex (Table II).

In order to demonstrate the reproducibility of the assay, the BA/DL and CD/DL assays were performed a total of 23 times over a period of 12½ months on the same concentration of a single DNA sample from a 15 week-old rat (#6776). The C_T values for these experiments are shown in Figure 4 and the ΔC_T values in Figure 5. Table III gives the mean and standard deviations for these results.

There was a significant linear trend in the ΔC_T values for the BA/DL measurement based on the day the experiment was done, with higher values associated with experiments done on

later dates. This linear trend explained roughly 23% of the total variability in the actin ΔC_T measurements. The trend is characterized as an average 0.0039 unit increase in the ΔC_T values for each additional day after the first. For the CD/DL measurements, the trend in ΔC_T versus day of the experiment had a more pronounced non-linear trend. However, the linear trend still explains roughly 14% of the overall variability in the ΔC_T values. The linear trend shows an average 0.0033 unit increase in the ΔC_T values for each additional day. The amount of overall variability in the ΔC_T values was higher for the second half of the experiments as compared with the first half. For both measurements the standard deviation of the results from the second half of the experiment was nearly twice as large as that of the first half. This could indicate some degradation or problems with uniform mixing in the later experiments. Furthermore, differential changes in the nuclear versus mitochondrial DNA will have a significant effect on the BA/DL experiments. Measurements of D-loop were made for both the BA/DL and CD/DL experiments. The correlation between the two different sets of measurements of D-loop was 0.903. This indicates good reproducibility on a single day and between the two types of assays, i.e. whether the D-loop was multiplexed with BA or CD, the same result was still obtained. The correlation between ΔC_T for BA/DL and ΔC_T for CD/DL is 0.409.

Finally, a series of rats of different ages (3 days to 23 months) was studied to determine changes in mtDNA copy number and changes in the frequency of the mtDNA deletion with age. Figures 6 and 7, respectively, show the results of these experiments based on $2^{-\Delta\Delta C_T}$. The frequency of mtDNA deletion increased 2.5 fold with age while copy number decreased rapidly over the first 3 months of age and then climbed slowly.

The ΔC_T measurements not surprisingly show a pattern that is roughly opposite that of the $2^{-\Delta\Delta C_T}$ measurements, with a decay over time. For the BA/DL data, this decay did not begin

until an age of 45 days. This possibly coincides with sexual maturity. The negative exponential model provided a good fit to the data and allowed for an estimate of the amount of time to lose half of the remaining amount. For the CD/DL data, the time to lose half of the remaining amount was estimated at 184.3 days and the asymptotic value for ΔC_T was 2.0. For the BA/DL data, using the measurements with age >45 days the amount of time to lose half of the remaining amount was estimated at 19.8 days and the asymptotic value for ΔC_T was 7.2.

DISCUSSION

Recently several methods have become available to quantitatively measure RNA or DNA levels using faster, more sensitive, real-time (non-endpoint), non-radioactive methods. These involve PCR monitored continuously by fluorescence as the reaction proceeds. The most popular method uses the TaqMan system of Applied Biosystems (ABI). In TaqMan, a probe specific for the gene of interest is created with a fluorescent dye at the 5' end and a quencher at the 3' end. As the specific PCR for the gene of interest progresses, the 5' exonuclease of the polymerase cleaves more and more free fluorescent dye from the probe that is then quantified. The amount of mRNA or DNA for a specific gene in the experimental sample can be quantified absolutely using a standard curve made with solutions with a known number of copies, quantified relative to a known sample, or quantified comparatively in the same reaction to another gene (an endogenous control). In the first two cases, separate amplifications of an endogenous control are needed to control for RT-efficiency and the amount of cDNA added to the reaction, while in the latter, the reactions for the control and experimental gene can be multiplexed. Our method simultaneously quantifies mtDNA and the common deletion rapidly and economically.

These experiments show that for the control liver DNA, the mean BA/DL ΔC_T was 8.0 which indicates that (assuming 100% efficiency of both PCRs) the mitochondrial sequence is present in ~256 more copies than the actin sequence ($=2^{\Delta C_T} = 2^{10} =1000$) or 512 copies/cell assuming the actin sequence is present in 2 copies. Liver has an average of 1300 mitochondria per cell, with 8-10 copies of mtDNA/mitochondria, giving 13,000 mitochondrial DNA copies/cell (Loud, 1968). However, calculating copy number from several published reports

gives lower numbers. For example, Gadelata et al. (1992) report that rats have 2.30ug mitochondrial DNA per mg of genomic DNA; this results in a copy number of ~860 in rat. Filser et al. (1997) reported 0.039% mtDNA which results in 146 copies/cell. Our calculated value is quite low compared to the value reported for liver but near those in Gadelata et al. (1992) and Filser et al. (1997). The low values reported here for copy number could be a result of actin pseudogenes in the rat genome; our calculation assumes 2 copies/cell but if there are 4 or 6 nuclear copies then the result should be multiplied by 2 or 3. Locus Link lists 6 actin-like genes in the rat. A Blast search, however, came up with a match to only the β-actin sequence. Further sequencing of the rat genome could reveal additional pseudogenes in the future. It must be pointed out that this comparative C_T method is not meant to give absolute quantitation but only relative quantitation between experimental conditions.

The results in Figure 6A show interesting changes in mitochondria copy number with age. At a young age (3 days to 60 days), mtDNA copy number is decreasing rapidly but then it begins a slow rise to 600 days. Others usually have found a slow rise with aging in mammals (Wong and Bai, 2002; Lee et al., 1998; Barrientos et al., 1997; Heerdt and Augenlicht, 1990). For example, Lee et al. (1998) found an increase of mtDNA copy number with aging in human lung, and Barrientos et al. (1997) found a 1.6 fold increase in 80 year olds versus 20 year olds. Heerdt and Augenlicht (1990) reported an increase in human mitochondrial DNA with development. Using hybridization to the COIII gene to mitochondrial DNA from liver they found a level of ~3 at 22 weeks gestation, ~5.5 at 32 weeks gestation and ~12 in two adults. Wong and Bai also report an increase in mtDNA from birth to 5 years of age in humans in muscle (2002). However, Barazzoni et al. (2000) found that 27mo old rats had only 50% of the mtDNA as 6mo old rats. Based upon *in vitro* studies, Tang et al. (2000) speculated that mtDNA

copy number is inversely proportional to the size of the mtDNA, i.e. cells keep a constant mass of mtDNA, not a constant number of genomes.

In terms of an "absolute" value for the frequency of the mtDNA deletion, the ΔC_T for the CD/DL experiment of 5.16 indicates that the common deletion is 0.028 (2.8%) of the total mitochondrial sequences [$1/2^{\Delta Ct} = 1/2^{5.16} = 1/35.75$], again assuming 100% efficiency for both reactions. The findings of other groups with regard to the frequency of mitochondrial DNA deletions are very variable, undoubtedly a result of the many experimental methods utilized as well as the tissues studied (Table IV). The numbers found here are higher than most of those reported, although similar to Filser et al. (1997) and Edris et al. (1994). In human liver, Wei et al. (1996) found a frequency of 0.00076% for 20-29 year olds and a frequency of 0.076% in 70-79 year olds, while Lee et al. (1994) found a frequency of 0.0076% in 70 year olds. Our results (Figure 6B) clearly confirm the reported increase in deletions in older animals.

In conclusion, we have developed an assay to detect both mtDNA copy number and mtDNA deletion frequency in the rat. We have shown that the assay is robust and reproducible and have demonstrated the expected results with aging. This assay should be useful to study the effects of drugs, diet or environmental changes on mitochondrial DNA.

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Table I. Sequences of the TaqMan primers and probes

Primer/Probe	Sequence (5'-3')
Mitochondrial D-loop	
Forward	GGTTCTTACTTCAGGGCCATCA
Reverse	GATTAGACCCGTTACCATCGAGAT
Probe	6FAM-TTGGTTCATCGTCCATACGTTCCCCTTA-TAMRA
Mitochondrial deletion	
Forward	AAGGACGAACCTGAGCCCTAATA
Reverse	CGAAGTAGATGATCCGTATGCTGTA
Probe	VIC-TCACTTTAATCGCCACATCCATAACTGCTGT-TAMRA
β-actin	
Forward	GGGATGTTTGCTCCAACCAA
Reverse	GCGCTTTTGACTCAAGGATTTAA
Probe	VIC-CGGTCGCCTTCACCGTTCCAGTT-TAMRA

Table II. Primer concentration experiments

β-actin Primer	D-loop primer	β-actin Ct	D-loop C_T	ΔC_T
200nM	100nM	27.55	18.54	9.01
200nM	50nM	27.09	19.03	8.06
200nM	--	27.45		
100nM	200nM	28.48	18.38	10.1
50nM	200nM	29.95	18.45	11.5
--	200nM		18.42	

Common deletion	D-loop primer	CD C_T	D-loop C_T	ΔC_T
200nM	200nM	23.54	18.43	5.11
200nM	100nM	22.48	18.31	4.17
200nM	50nM	22.82	18.74	4.08
200nM	--	23.32		
100nM	200nM	25.36	18.36	7.00
50nM	200nM	28.41	18.22	10.10

Table III. Results of the replicate experiments

ΔC_T	measure	all days	days<=180	days>180
actin	mean	5.250	4.772	5.556
	std	0.928	0.514	1.010
deletion	mean	8.131	7.888	8.288
	std	1.000	0.506	1.201

Table IV. Mitochondrial deletion frequency in rats reported by others

Tissue	Deletion frequency in young rats	Deletion frequency in older rats	Reference
brain (striatum)	0.0003% (6mo)	0.0075% (22mo)	(21)
brain (dorsal root)	0.00005% (3mo)	0.015% (24mo)	(18)
Liver	0.0005% (7mo)	0.02% (27mo)	(16)
Liver	0.05% (6mo)	0.32% (24mo)	(20)
Liver	0.001% (6 mo)	0.5% (24mo)	(17)
Liver	ND	1.7% (20 mo)	(36)
Liver	ND	1.88% (~24mo)	(6)

FIGURE LEGENDS

Fig. 1. TaqMan Probes and Primers. The sequences of the rat mitochondrial DNA for both the mitochondrial deletion region (before and after deletion) and the D loop region as well as the genomic beta actin gene (exon 6) are shown with the TaqMan primers and probes highlighted.

Fig. 2. Slopes of C_T versus log (starting DNA). C_T was measured for 5 dilutions of input DNA for each primer and probe set separately [beta-actin (BA), D-loop (DL) and common deletion (CD)]. C_T is plotted versus the log of the input DNA. Slopes of the trend line for each probe were determined. A primer/probe set with 100% efficiency would have a slope of –3.33.

Fig. 3. Slopes of del C_T versus input DNA for the multiplex PCR reactions. The multiplex reactions of beta-actin with D-loop probe/primers to measure mtDNA copy number (BA/DL) and common deletion with D-loop probe/primers to measure the frequency of the common mitochondrial deletion (CD/DL) were performed on 5 dilutions of input DNA. The del C_T is plotted versus the log of input DNA. Slopes should be <-0.1 for a good probe/primer set.

Fig. 4. C_T values for 23 replicate experiments. Experiments to measure the C_T for BA and DL in a multiplex BA/DL assay and the C_T for CD and DL in a CD/DL assay were performed 23 times on DNA from a single rat liver (rat #6776) over a 370 day period. C_T is plotted versus the day since the first experiment (day 1).

Fig. 5. Del C_T values for 23 replicate experiments. The BA/DL del C_T (mitochondrial copy number) and the CD/DL del C_T (frequency of the common deletion) are plotted for the experiments in Figure 4. Del C_T is plotted versus the day since the first experiment (day 1).

Fig. 6. Changes in mt DNA copy number with age. DNA was isolated from the liver of rats from newborn to 700 days old. The newborn BA/DL del C_T(mitochondrial copy number) was taken as the reference point for a del del C_T to calculate $2^{-deldelC}{}_T$ that is plotted versus the days in age of the rats.

Fig. 7. Changes in the frequency of mitochondrial deletion with age. DNA was isolated from the liver of rats from newborn to 700 days old. The newborn CD/DL del C_T (mitochondrial deletion frequency) was taken as the reference point for a del del C_T to calculate $2^{-deldelC}{}_T$ that is plotted versus the days in age of the rats.

Mitochondrial Deletion

```
attcacacac caaaaggacg aaCCTGAGCCC CTAATAATtg tatccctaat .. .. .. ..   acgtgtaaca ccaacgCCTG AGCCCTAATA ATcactttaa tcgccacatc
8081                                                                 12921

Deletion                attcacacac caaaaggacg aaCCTGAGCC CTAATAATAT cactttaatc gccacatcat aactgctgt gtacagcaac ggatcatct acttcgtcac catgacaa
```

NNN - mitochondrial repeat
→ - PCR primers
nnn - TaqMan probe

Mitochondrial D Loop

```
ctgaaacttt accaggcatc tggttcttac ttcagggcca tcaattggtt catcgtccat acgttcccct taaataagac atctcgatgg taacgggtct aatcagccca tgatcaacat
15751
```

→ - PCR primers
nnn - TaqMan probe

Genomic Beta Actin (exon 6) (probe is complement of shown strand)

```
ttttgttttg gcgcttttga ctcaaggatt taaaaactgg aacggtgaag gcgaccgcag ttggttggag caaacatccc ccaaagttct acaatgtggc
2991
```

→ - PCR primers
nnn - TaqMan probe

Fig.1. Probes and Primers

Fig. 2. Slopes of Ct versus log [input DNA]

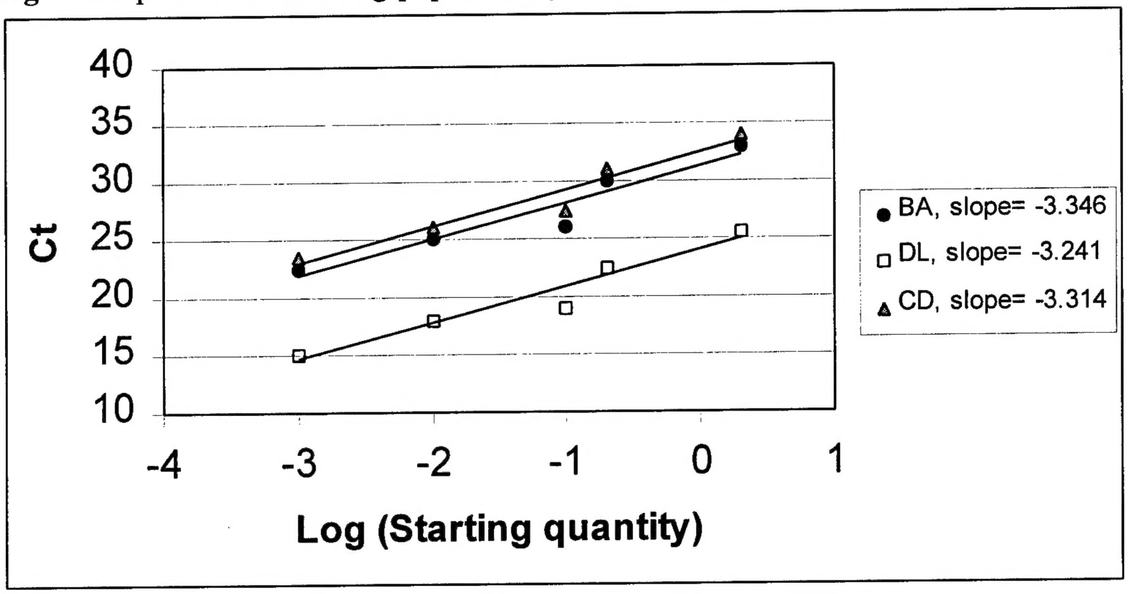

Fig. 3. Slopes of Ct versus [DNA] for Multiplex PCR reaction

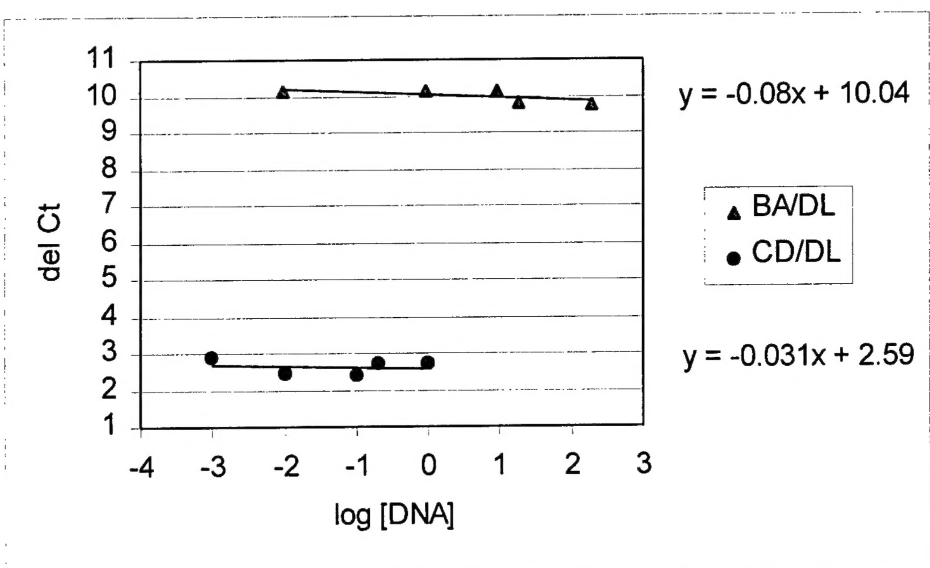

Fig. 4. Ct values for 23 replicate experiments